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In Vitro and In Vivo Activities of T-705 against Arenavirus and Bunyavirus Infections▽

Brian B. Gowen,[1]* Min-Hui Wong,[1] Kie-Hoon Jung,[1] Andrew B. Sanders,[1]† Michelle Mendenhall,[1] Kevin W. Bailey,[1] Yousuke Furuta,[2] and Robert W. Sidwell[1]

Institute for Antiviral Research and Department of Animal, Dairy, and Veterinary Sciences, Utah State University, Logan, Utah,[1] and Research Laboratories, Toyama Chemical Company, Ltd., Toyama, Japan[2]

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There is a need for the development of effective antivirals for the treatment of severe viral diseases caused by members of the virus families *Bunyaviridae* and *Arenaviridae*. The pyrazine derivative T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) has demonstrated remarkable antiviral activity against influenza virus and, to a lesser degree, against some other RNA viruses (Y. Furuta, K. Takahashi, Y. Fukuda, M. Kuno, T. Kamiyama, K. Kozaki, N. Nomura, H. Egawa, S. Minami, Y. Watanabe, H. Narita, and K. Shiraki, Antimicrob. Agents Chemother., 46:977–981, 2002). Here, we report that T-705 is highly active against a panel of bunyaviruses (La Crosse, Punta Toro, Rift Valley fever, and sandfly fever viruses) and arenaviruses (Junin, Pichinde, and Tacaribe viruses) by cytopathic effect and virus yield reduction cell-based assays. The 50% effective concentrations for T-705 ranged from 5 to 30 μg/ml and 0.7 to 1.2 μg/ml against the bunyaviruses and arenaviruses examined, respectively. We also demonstrate that orally administered T-705 is efficacious in treating Punta Toro virus in the mouse and hamster infection models, as well as Pichinde virus infection in hamsters. When administered twice daily for 5 to 6 days, beginning 4 h pre- or 24 h post-Punta Toro virus challenge, a 30-mg/kg of body weight/day dose provided complete protection from death and limited viral burden and liver disease. A dose of 50 mg/kg/day was found to be optimal for treating Pichinde infection and limiting viral replication and disease severity. In general, T-705 was found to be more active than ribavirin in cell-based assays and in vivo, as reflected by substantially greater therapeutic indexes. Our results suggest that T-705 may be a viable alternative for the treatment of life-threatening bunyaviral and arenaviral infections.

Several members of the RNA virus families *Arenaviridae*, *Bunyaviridae*, *Filoviridae*, and *Flaviviridae* can cause viral hemorrhagic fever (VHF). These viruses are highly feared as a result of the extreme morbidity and mortality associated with the severe forms of disease they can cause. Currently, there is a paucity of safe and effective antivirals for the treatment of VHF caused by these viruses. Ribavirin has proven to be effective against several of the arenaviral and bunyaviral hemorrhagic fevers (4, 6, 12–15) but is approved only for compassionate use under investigational new drug protocols due to concerns with dose-related hemolytic anemia and potential teratogenicity (2, 23). To date, there are no United States FDA-approved antivirals for the treatment of VHFs. The threat of intentional release of VHF agents by terrorist groups and naturally occurring outbreaks in various regions of the world underlines the need for the development of novel therapeutics and disease management strategies.

T-705 (6-fluoro-3-hydroxy-2-pyrazinecarboxamide) is a novel pyrazine derivative originally described in 2002 as a compound with potent anti-influenza activity in cell-based assays and infected mice (7, 8, 20, 24). It is acted upon by host cell enzymes, and its ribophosphorylated product functions as a purine nucleotide analog that is highly selective for influenza virus polymerase (8). Evidence suggests that T-705 acts in a different manner than ribavirin, since it only weakly inhibits IMP dehydrogenase (IMPDH) and does not measurably disrupt RNA and DNA synthesis, both of which likely contribute to the observed lack of toxicity (8). T-705 has also demonstrated in vitro activity against several other RNA viruses. Although not as potent as seen with influenza virus, the T-705 inhibitory effects against poliovirus, rhinovirus, and respiratory syncytial virus (RSV) are moderate, and lack of activity against several DNA viruses suggests specificity for RNA viruses (7). In light of these findings, the potential antiviral activity of T-705 warrants examination against other RNA virus pathogens.

Reported here are the results of a series of studies investigating the in vitro and in vivo efficacies of T-705 against a group of bunyaviruses and arenaviruses that serve as surrogate models for the more biohazardous members of their respective families. Ribavirin, known to be active against all of the tested viruses, was included for comparison.

MATERIALS AND METHODS

Cells and animals. The monkey kidney cell lines Vero, Vero 76, LLC-MK_2, and B-SC-1 were purchased from the American Type Culture Collection (ATCC) (Manassas, VA) and maintained in minimal essential medium (MEM) supplemented with 0.18% $NaHCO_3$ and 10% fetal bovine serum (HyClone, Logan, UT). The medium for B-SC-1 was further supplemented with 0.1 mM nonessential amino acids (Invitrogen, Carlsbad, CA) and 1 mM sodium pyruvate (Sigma, St. Louis, MO). Female 12- to 14-g C57BL/6 mice and 90- to 100-g golden Syrian hamsters were obtained from Charles River Laboratories (Wilmington, MA). The mice and hamsters were acclimated for 3 to 6 days prior to use. Animal procedures complied with USDA and Utah State University Institutional Animal Care and Use Committee guidelines.

* Corresponding author. Mailing address: Institute for Antiviral Research, Utah State University, Logan, UT 84322. E-mail: bgowen@cc.usu.edu. Phone: (435) 797-3112. Fax: (435) 797-3959.

† Present address: Department of Pediatrics, University of Utah, Salt Lake City, UT.

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TABLE 1. In vitro inhibitory effects of T-705 and ribavirin against bunyaviruses[a]

Virus	Strain	T-705[b]			Ribavirin[b]		
		CC_{50} ± SD	EC_{50} ± SD	SI[c]	CC_{50} ± SD	EC_{50} ± SD	SI
LACV		>6,365 ± 0	32 ± 13	>199	3,595 ± 864	70 ± 49	51
PTV	Adames	>6,365 ± 0	191 ± 32	>33	3,681 ± 360	172 ± 90	21
RVFV	MP-12	>6,257 ± 185	32 ± 6	>196	>3,714 ± 659	53 ± 16	>70
SFNV	Naples	>6,365 ± 0	115 ± 166	>55	>2,989 ± 901	90 ± 49	>33

[a] Data are the means and standard deviations from three or four separate experiments in Vero 76 cells.
[b] CC_{50} and EC_{50} values are in μM.

Viruses. La Crosse virus (LACV), a clinical isolate; sandfly fever virus (SFNV), Naples strain; and Tacaribe virus (TCRV), strain TRVL 11573, were all purchased from the ATCC. Punta Toro virus (PTV), Adames strain, was obtained from Dominique Pifat of the U.S. Army Medical Research Institute for Infectious Diseases, Ft. Detrick (Frederick, MD). The Rift Valley fever virus (RVFV) vaccine strain, MP-12, and the Junin virus (JUNV) vaccine strain, Candid 1, were kindly provided by Robert Tesh (World Reference Center for Emerging Viruses and Arboviruses, University of Texas Medical Branch, Galveston). Pichinde virus (PICV), strain An 4763, was provided by David Gangemi (Clemson University, Clemson, SC).

Test materials. T-705 was provided by the Toyama Chemical Company, Ltd. (Tokyo, Japan). For in vitro testing, T-705 was dissolved in dimethyl sulfoxide and further diluted in MEM (HyClone, Logan, UT) so that the highest test concentration did not exceed 1% dimethyl sulfoxide. For in vivo delivery into mice and hamsters, T-705 was suspended in 0.4% carboxymethylcellulose (CMC). Ribavirin was supplied by ICN Pharmaceuticals, Inc. (Costa Mesa, CA). It was dissolved in MEM for in vitro studies and in sterile saline solution for in vivo administration.

In vitro antiviral testing. Viruses were diluted in culture medium containing 2% fetal bovine serum to a cell culture 50% infectious dose ($CCID_{50}$) that produced maximal cytopathic effect (CPE) by visual examination in preliminary virus titration experiments. Half-log dilutions of T-705 and ribavirin were added to test wells at the time of infection. For toxicity determinations, drugs were added in the absence of viral challenge. Plates were incubated at 37°C and 5% CO_2 until virus-infected control wells were observed to have maximal viral CPE, at which time the plates were scored visually for CPE and toxicity. The median effective concentration (EC_{50}) and the concentration that reduced cell viability by 50% (CC_{50}) were determined by regression analysis, and selectivity index (SI) values were calculated as follows: $SI = CC_{50}/EC_{50}$. Virus yield reduction data were determined as the concentration of drug that reduced the virus yield by 1 $\log_{10}$ unit (EC_{90}) based on regression analysis.

Studies with bunyaviruses (LACV, PTV, SFNV, and RVFV) were done using Vero 76 cells (~90% confluence) plated in 96-well, half-growth-area, clear-bottom, white opaque polystyrene microplates. Following visual CPE and toxicity determination 3 to 5 days postinfection, the cell viability across the entire plate was measured by assaying for the presence of ATP using the Cell-Titer Glo system from Promega (Madison, WI). Luminescence was read on an LB960 Centro luminometer from Berthold Technologies (Oak Ridge, TN). Luminescence values were expressed as percentages of untreated, uninfected controls, and EC_{50}, CC_{50}, and SI values were calculated as described above.

Due to the extended incubation times required for the development of CPE (7 to 8 days) following infection with several arenaviruses (JUNV and PICV), ATP production in cell cultures slows considerably, limiting the utility of the Cell-Titer Glo assay. Thus, the neutral red (NR) dye uptake method for measuring cell viability was used for the arenavirus studies. Drug activity studies with the less cytopathic arenaviruses were conducted using subconfluent (50 to 70%) Vero cells plated in standard 96-well microplates, since more pronounced CPE was observed under these conditions. Following visual analysis of CPE and toxicity, infected cells and controls were incubated with 0.034% NR solution for 2 h at 37°C and 5% CO_2. After incubation, the NR was removed and the wells were rinsed twice with phosphate-buffered saline. The plates were allowed to dry completely prior to 30-minute extraction of the vital dye with absolute ethanol buffered with Sorenson's citrate buffer. Samples were read at 540 nm on a BioTek EL 800 microplate reader (BioTek, Winooski, VT), and the absorbance values were expressed as percentages of untreated, uninfected controls, which took up maximal dye.

Mouse and hamster challenge studies. Groups of 15 mice or hamsters (25 to 30 for the placebo groups) were treated with various doses and schedules of T-705, ribavirin, or placebo, starting prior to or after subcutaneous inoculation with 50, 500, or 5,000 PFU of PTV for mouse challenges and 50 PFU for hamsters. For PICV challenge studies, 65 PFU of virus was inoculated by bilateral intraperitoneal injection. Drugs were administered twice a day for 5 to 7 days by oral-gavage (p.o.) administration. Five animals from each group (10 for the placebo) were sacrificed on day 3 or 4 of infection for PTV experiments and day 7 for PICV studies. Livers were scored on a scale of 0 to 4 for hepatic icterus, 0 being normal and 4 being maximal yellow coloration. Serum was collected for assaying alanine aminotransferase (ALT) activity, and virus titers were determined for both liver and serum samples as described below. The remaining animals in each group were observed for 21 to 28 days. Three sham-infected animals were included as normal controls to establish baselines for all test parameters. In initial studies, toxicity associated with test materials was evaluated in uninfected animals. All tested doses were found to be well tolerated, as indicated by normal weight gain, activity, and appearance.

Evaluation of liver and serum virus burdens. Virus titers were determined using an infectious cell culture assay as previously described (9, 22). Briefly, specific volumes of liver homogenate or serum were serially diluted and added to triplicate wells of $LLC\text{-}MK_2$ (PTV) or B-SC-1 (PICV) cell monolayers in 96-well microplates. The viral CPE was determined 6 to 8 days post-virus exposure, and the 50% endpoints were calculated as described previously (17). The assay detection range was 2.8 to 9.5 $\log_{10}$ $CCID_{50}$/0.1 g of liver or 0.1 ml of serum. In samples presenting with no detectable liver or serum virus, a value of <2.8 $\log_{10}$ was assigned. Therefore, a mean virus titer value preceded by "<" indicates that at least one of the samples had undetectable levels of virus and is likely an overestimate of the actual mean viral load. Conversely, in cases where virus exceeded the detection range, a value of >9.5 $\log_{10}$ was assigned. Thus, samples with a value preceded by ">" are likely an underestimate of the actual viral load. For statistical analysis, values of 2.8 or 9.5 $\log_{10}$ were assigned as needed for samples with undetectable or saturated virus levels, respectively.

ALT measurement. Serum ALT release serves as an indicator of liver damage and malfunction. ALT activity was measured by a kinetic assay using the ALT (SGPT) Reagent Set from Pointe Scientific, Inc. (Lincoln Park, MI). The reagent volumes were modified for use with 96-well microplates following the manufacturer's recommendations.

Statistical analysis. Fisher's exact test (two-tailed) was employed to evaluate increases in total numbers of survivors. The log rank test was used for comprehensive survival analysis using JMP statistical software (SAS, Cary, NC). The Mann-Whitney test (two-tailed) was performed to analyze the differences in mean days to death (MDD), virus titers, serum ALT levels, and liver scores.

RESULTS

In vitro antiviral activity of T-705 against bunyaviruses. The antiviral activity of T-705 was first evaluated in parallel with ribavirin against several known human pathogens belonging to the family *Bunyaviridae*. Inhibitory activity, represented as EC_{50}, against LACV, PTV, RVFV, and SFNV ranged from 32 to 191 μM for T-705 and 53 to 172 μM for ribavirin, included for comparison as a positive control (Table 1). Despite comparable mean EC_{50} values, ribavirin was found to be considerably more toxic than T-705, as indicated by the lower CC_{50} values (Table 1). As a result, the SI values for T-705 against LACV, PTV, RVFV, and SFNV were >3.8-, >1.6-, 2.8-, and 1.7-fold greater than for ribavirin. The antiviral activity of T-705 was verified by visual CPE reduction assessment (data

TABLE 2. Effects of oral T-705 treatment[a] on mice challenged with PTV

Drug	Dosage (mg/kg/day)	Survived/ total	MDD[b] ± SD	Mean virus titer[c,d] ± SD		Mean ALT[c,e] ± SD	Mean liver score[c,f] ± SD
				Liver	Serum		
T-705	200	4/9[g]	8.0 ± 2.8[g]	<2.8 (0)	<2.8 (0)	37 ± 7	0.0 ± 0.0[h]
	100	10/10[i]	>21.0	<2.8 (0)	<2.8[g] (0)	33 ± 14[g]	0.3 ± 0.3[i]
	50	9/9[i]	>21.0	<2.8 (0)	<3.1 ± 0.7[g] (10)	41 ± 26[g]	0.3 ± 0.3[i]
	25	10/10[i]	>21.0	<2.8 (0)	<3.3 ± 1.1[g] (20)	69 ± 49[g]	0.6 ± 0.7[i]
Ribavirin	75	10/10[i]	>21.0	<2.8 (0)	<3.3 ± 0.8[g] (20)	38 ± 33[g]	0.9 ± 0.4[i]
0.4% CMC		1/20	4.5 ± 0.6	<3.6 ± 1.1 (60)	6.9 ± 1.2 (100)	3789 ± 713	3.6 ± 0.2
Sham infected		3/3				20 ± 18	0.0 ± 0.0

[a] Twice daily for 5 days beginning 4 h pre-virus inoculation with 500 PFU of PTV.
[b] MDD of mice dying prior to day 21.
[c] Determined on day 4 of infection; 5 mice per group (10 in the placebo group). Due to mortality prior to time of sacrifice, serum titers and ALT could not be determined for two and seven of the mice from the 200-mg/kg/day T-705 group and the placebo group, respectively.
[d] Log_{10} $CCID_{50}$/0.1 g of liver or ml of serum. The percentage of animals presenting with detectable virus levels is indicated in parentheses.
[e] Measured in international units per liter.
[f] Score of 0 (normal liver) to 4 (maximal discoloration).
[g] $P < 0.05$ compared to 0.4% CMC-treated controls.
[h] $P < 0.01$ compared to 0.4% CMC-treated controls.
[i] $P < 0.001$ compared to 0.4% CMC-treated controls.

not shown) and corroborated by experiments measuring virus yield reduction (EC_{90} values of 7, 20, 6, and 3 μM for LACV, PTV, RVFV, and SFNV, respectively). The data indicate that T-705 is inhibitory against the tested bunyaviruses, may possess greater activity than ribavirin, and appears to be less cytotoxic to cell cultures.

In vivo efficacy of T-705 against PTV infection in mice. We next evaluated T-705 in the weanling mouse PTV infection model of acute phleboviral disease. Doses of 200, 100, 50, and 25 mg/kg of body weight/day were selected based on previous studies in mice (7) and were given p.o. twice per day for 5 days. As shown in Table 2, with the exception of the highest dosage, T-705 was highly effective, as it offered complete protection against a highly lethal PTV challenge dose. Since no apparent weight loss or illness was evident with any of the tested T-705 doses examined in a parallel toxicity evaluation, we suspect that in the face of viral infection, trauma due to the larger-diameter p.o. needle required for delivery of the highly viscous 200-mg/kg dose contributed to the observed reduction in efficacy. Significant protection was still observed with the high-dose T-705 preparation despite only four of the nine mice surviving the infectious challenge. Moreover, the mice that died survived appreciably longer (3.5 days) than those receiving vehicle only. For comparison, ribavirin was also evaluated by the same route and schedule at a dosage of 75 mg/kg/day and, as expected, provided 100% protection.

Analysis of liver and systemic viral loads assessed on day 4 of the infection demonstrated that T-705 was equally as effective as, if not better than, ribavirin in thwarting viral replication (Table 2). Comparable reductions in liver disease were also evident, as ALT levels and liver scores were very similar across treatment groups. It should also be noted that serum viral burden and ALT values for the placebo group are more than likely an underestimate of the severity of infection and disease, since serum could be collected from only 3 of the 10 mice due to mortality prior to the time of sacrifice (Table 2). This was also the case for the 200-mg/kg/day group, in which serum samples could be collected from only three of the five animals. Thus, p.o. administration of 25 to 100 mg/kg/day of T-705 was found highly effective in protecting mice challenged with a 95% lethal dose (LD_{95}) inoculum of PTV, with a dramatic reduction in the viral load and liver disease comparable to that of the positive control drug, ribavirin.

Protection limits of T-705 in a mouse PTV infection model. In the initial trial with T-705, the lower limit of protection was not defined, as the 25-mg/kg/day dose offered complete protection. Therefore, a second study was conducted administering 30, 10, and 3 mg/kg/day to determine the limits of protection in the PTV mouse infection model. As shown in Table 3, the 10-mg/kg/day dosage afforded good protection in the context of survival, as 90% of the animals were protected. However, efficacy was entirely lost at the lowest treatment dose of 3 mg/kg/day with no appreciable increase in the MDD of animals that succumbed to the infection. As expected, the positive control treatment, 75 mg/kg/day of ribavirin, provided complete protection. The lowest effective dose of 10 mg/kg/day was considerably less than the LD_{50} of T-705 (707 mg/kg/day) determined in weanling C57BL/6 mice.

When the various virologic and liver disease parameters were assessed, liver virus was undetectable in T-705-treated mice, regardless of the dose (Table 3). In contrast, 30% of the mice in the placebo group and 20% of the mice in the ribavirin group presented with detectable levels of liver virus. In the context of serum virus titers, the burden was reduced in a dose-responsive fashion with highly significant reductions observed with both the 30- and 10-mg/kg/day doses of T-705 (Table 3). In the second experiment, T-705 was not as effective as ribavirin in limiting the systemic viral burden. Consistent with the pattern of systemic viral load, a clear dose-response was seen in mean serum ALT levels for T-705 (Table 3). Both the 30- and 10-mg/kg/day doses of both drugs significantly reduced ALT levels compared to the placebo, but only the mice in the higher-dose group had baseline ALT values comparable to those of the sham-infected and the ribavirin-treated mice, suggesting that little to no liver damage resulted. In agreement with the lack of serum virus burden with ribavirin treatment, ribavirin completely prevented liver discoloration, whereas the two highest doses of T-705 had only a slight yet significant impact on reducing liver scores (Table 3). It is important to note that ribavirin was used at a considerably higher

TABLE 3. Determination of protection limits of oral T-705 treatment[a] for mice challenged with PTV

Drug	Dosage (mg/kg/day)	Survived/ total	MDD[b] ± SD	Mean virus titer[c,d] ± SD		Mean ALT[c,e] ± SD	Mean liver score[c,f] ± SD
				Liver	Serum		
T-705	30	10/10[i]	>21.0	<2.8 (0)	<5.2 ± 1.4[i] (80)	9 ± 8[i]	2.0 ± 0.6[h]
	10	9/10[i]	5.0	<2.8 (0)	6.2 ± 0.4[h] (100)	501 ± 296[h]	2.5 ± 0.0[g]
	3	1/10	4.9 ± 1.1	<2.8 (0)	7.2 ± 0.5 (100)	2,461 ± 921	2.8 ± 0.3
Ribavirin	75	10/10[i]	>21.0	<2.9 ± 0.2 (20)	<2.8 (0)[i]	13 ± 6[i]	0.0 ± 0.0[i]
0.4% CMC		0/20	5.0 ± 0.9	<4.2 ± 1.5 (30)	7.2 ± 0.4 (100)	3,571 ± 1,476	3.3 ± 0.6
Sham infected		3/3				13 ± 5	0.0 ± 0.0

[a] Twice daily for 5 days beginning 4 h pre-virus inoculation with 500 PFU of PTV.
[b] MDD of mice dying prior to day 21.
[c] Determined on day 3 of infection; 5 mice per treatment group (10 in the placebo group).
[d] Log_{10} $CCID_{50}$/0.1 g of liver or ml of serum. The percentage of animals presenting with detectable virus levels is indicated in parentheses.
[e] Measured in international units per liter.
[f] Score of 0 (normal liver) to 4 (maximal discoloration).
[g] $P < 0.05$ compared to 0.4% CMC-treated controls.
[h] $P < 0.01$ compared to 0.4% CMC-treated controls.
[i] $P < 0.001$ compared to 0.4% CMC-treated controls.

dose as the positive control drug to ascertain the treatability of the infectious challenge. Taking these data together, although the 10-mg/kg/day dose of T-705 was less effective at limiting disease parameters, in the context of survival, it was equally as effective as the 30-mg/kg/day treatment.

In vivo comparison of T-705 and ribavirin anti-PTV activities in mice. Ribavirin has been the subject of considerable testing efforts against PTV and RVFV, with demonstrated efficacy against both (16, 21). To this end, it is routinely used as the positive control drug for antiviral studies conducted in the PTV mouse infection model. The data presented in Table 2 suggest that T-705 has potent antiviral activity similar to that of ribavirin. Therefore, the LD_{50}s for both drugs in weanling C57BL/6 mice were determined. When ribavirin was administered p.o. for 5 days, twice per day, its LD_{50} (730 mg/kg/day) was comparable to that of T-705 (707 mg/kg/day). Next, we directly compared equivalent optimal and suboptimal doses and schedules of T-705 and ribavirin against various log_{10} dilutions of challenge virus, starting treatment 24 h postinfection. The highest infectious dose of 5×10^4 PFU resulted in 100% mortality, with the majority of the placebo-treated animals dying rapidly by day 4 (Table 4). Although the results were not very dramatic when examined individually, the collective results comparing T-705 treatment to that with ribavirin suggests that T-705 may be slightly more efficacious at this challenge dose. Notably, with the challenge dose of 5×10^3 PFU, 90 and 40% of the mice treated with 30 and 10 mg/kg/day of T-705, respectively, survived compared to the ribavirin-

TABLE 4. Comparison of therapeutic T-705 and ribavirin treatment[a] for mice challenged with PTV

Treatment, PTV inoculum (PFU)	Dosage (mg/kg/day)	Survived/ total	MDD[b] ± SD	Mean virus titer[c,d] ± SD		Mean ALT[c,e] ± SD	Mean liver score[c,f] ± SD
				Liver	Serum		
T-705, 5,000	30	9/10[i]	5.0	<2.9 ± 0.2 (20)	<3.3 ± 1.2 (80)[g]	521 ± 438[g]	1.5 ± 0.8[g,j]
	10	3/10[g]	4.4 ± 0.5	<2.9 ± 0.3 (20)	<5.2 ± 1.8 (80)	2,274 ± 1,437	2.5 ± 0.8
Ribavirin, 5,000	30	9/10[i]	5.0	<2.9 ± 0.3 (20)	<4.2 ± 2.0 (60)	1,491 ± 790	3.3 ± 0.8
	10	2/10	4.0 ± 0.0	<2.8 (0)	5.9 ± 2.0 (100)	1,515 ± 848	3.5 ± 0.5
0.4% CMC, 5,000		0/20	4.1 ± 0.4	<3.3 ± 1.0 (40)	6.7 ± 0.8 (100)	4,044 ± 1,888	3.1 ± 0.8
T-705, 500	30	9/10[i]	5.0	<3.2 ± 0.5 (60)	<5.1 ± 1.4 (80)	900 ± 1744[g]	2.2 ± 0.3[h]
	10	4/10[h]	4.7 ± 0.8	<2.8 (0)	7.1 ± 0.5 (100)	3,302 ± 1,461	3.2 ± 0.6
Ribavirin, 500	30	7/10[i]	5.3 ± 1.5	<3.1 ± 0.4 (60)	<5.0 ± 1.4 (80)[g]	450 ± 583[h]	2.2 ± 0.9
	10	1/10	5.0 ± 0.9[h]	<2.9 ± 0.3 (20)	6.7 ± 0.7 (100)	2,150 ± 933	3.0 ± 0.5
0.4% CMC, 500		0/20	4.1 ± 0.2	<3.7 ± 1.7 (40)	6.9 ± 0.7 (100)	5,526 ± 2,031	3.0 ± 0.4
T-705, 50	30	10/10[i]	>21.0	<2.8 ± 0.1 (40)	<4.4 ± 1.4 (80)[h]	199 ± 180[h]	1.3 ± 0.4[h,j]
	10	0/10	5.8 ± 1.6[g]	<2.8 (0)	6.9 ± 0.4 (100)	3,273 ± 914[j]	2.5 ± 0.4
Ribavirin, 50	30	10/10[i]	>21.0	<2.8 ± 0.0 (20)	6.1 ± 0.4 (100)	131 ± 86[h]	2.2 ± 0.3
	10	2/9	5.3 ± 0.5[g]	<3.3 ± 0.8 (40)	<4.9 ± 2.0 (60)	846 ± 1,139	2.8 ± 1.3
0.4% CMC, 50		3/20	4.5 ± 0.5	<3.1 ± 0.8 (20)	6.8 ± 0.4 (100)	2,981 ± 1,917	2.6 ± 0.4
Sham infected		3/3				3 ± 3	0.0 ± 0.0

[a] P.o., twice daily for 5 days beginning 24 h post-virus inoculation.
[b] MDD of mice dying prior to day 21.
[c] Determined on day 3 of infection; five mice per treatment group.
[d] Log_{10} $CCID_{50}$/0.1 g of liver or ml of serum. The percentages of animals presenting with detectable virus levels are indicated in parentheses.
[e] Measured in international units per liter.
[f] Score of 0 (normal liver) to 4 (maximal discoloration).
[g] $P < 0.05$ compared to 0.4% CMC-treated controls.
[h] $P < 0.01$ compared to 0.4% CMC-treated controls.
[i] $P < 0.001$ compared to 0.4% CMC-treated controls.
[j] $P < 0.05$ compared to group receiving equivalent viral inoculum and dose of ribavirin.

TABLE 5. Effect of oral T-705 treatment[a] on hamsters challenged with PTV

Drug	Dosage (mg/kg/day)	Survived/ total	MDD[b] ± SD	Mean virus titer[c,d] ± SD		Mean ALT[c,e] ± SD	Mean liver score[c,f] ± SD
				Liver	Serum		
T-705	60	9/10[i]	6.0	<3.4 ± 1.3[g] (20)	<3.8 ± 2.2 (20)	17 ± 3	0.0 ± 0.0[i]
	30	9/10[i]	6.0	<3.5 ± 1.7[g] (40)	<4.5 ± 2.6 (40)	25 ± 18	0.0 ± 0.0[i]
	15	5/10[h]	5.4 ± 0.5	>6.9 ± 1.5 (100)	<4.1 ± 3.0 (20)	204 ± 441	0.5 ± 0.6
Ribavirin	30	7/10[i]	13.3 ± 2.1	<2.8[h] (0)	<2.8 (0)	13 ± 13	0.0 ± 0.0[i]
0.4% CMC		1/20	8.4 ± 4.1	<6.6 ± 2.2 (90)	<5.4 ± 3.4 (40)	1,228 ± 1,622	1.3 ± 0.7
Sham infected		3/3				28 ± 16	0.0 ± 0.0

[a] Twice daily for 6 days beginning 4 h pre-virus inoculation with 5 PFU of PTV.
[b] MDD of hamsters dying prior to day 21.
[c] Determined on day 4 of infection; 5 hamsters per group (10 in the placebo group).
[d] Log_{10} $CCID_{50}$/0.1 g of liver or ml of serum. The percentage of animals presenting with detectable virus levels is indicated in parentheses.
[e] Measured in international units per liter.
[f] Score of 0 (normal liver) to 4 (maximal discoloration).
[g] $P < 0.05$ compared to 0.4% CMC-treated controls.
[h] $P < 0.01$ compared to 0.4% CMC-treated controls.
[i] $P < 0.001$ compared to 0.4% CMC-treated controls.

treated groups, which had 70 and 10% survival (Table 4). Despite this, there was no evidence of T-705 having a greater antiviral effect at this intermediate challenge dose when viral loads and liver disease were evaluated, as it appeared that ribavirin was slightly better in that regard. Moreover, the differences in total numbers of survivors between respective treatment groups, at any PTV challenge dose, were not found to be statistically significant. At the lowest infectious challenge dose, despite there being some remarkable differences between the two drugs, there was no clear evidence of one being superior to the other (Table 4). Somewhat surprising was the low-dose T-705 therapy, where there were no survivors and excessively high ALT levels. In fact, at the 10-mg/kg/day dose, T-705-treated animals had, at all infectious doses, the highest ALT values. However, the liver scores did not reflect this indication of more pronounced liver disease, since the same trend was not observed (Table 4).

T-705 treatment of PTV infection in hamsters. Acute phleboviral infection can also be modeled in hamsters infected with PTV for the evaluation of antiviral therapies (10). Based on pathological studies, the hamster model may be more representative of human disease (1, 5). To further examine the antiviral activity of T-705 against PTV infection, we evaluated T-705 in the hamster infection model, with the highest test dose of 60 mg/kg/day being twice the effective amount for optimal efficacy in mice. Drug treatments in the hamster model were extended to 6 days. T-705 was highly effective at protecting hamsters from lethal PTV infection. As demonstrated in Table 5, the 60- and 30-mg/kg/day regimens of T-705 protected equally well (90% survival) against an infectious challenge dose that was 95% lethal in placebo-treated hamsters. The positive control, ribavirin, tested at 30 mg/kg/day, protected 70% of the hamsters. Although a clear decrease in antiviral activity was noted with the lowest treatment doses of T-705, it was still significantly refractory to viral challenge (50% survival rate) compared to the placebo-treated hamsters, as indicated by log rank analysis ($P > \chi^2$, 0.03) and Fisher's exact test (Table 5) ($P < 0.01$). The two highest doses of T-705 inhibited the viral burden and liver disease comparably, with the 15-mg/kg/day dose being less effective at limiting the liver virus burden and liver disease, as determined on day 4 of the infection (Table 5). Hamsters treated with ribavirin presented with undetectable levels of liver and serum virus and with no signs of liver disease. This was consistent with the apparent delay in disease progression in the three ribavirin-treated animals that died (MDD > 13 days). Collectively, the mouse and hamster PTV infection model data indicate that T-705 inhibits infection and disease effectively in both systems.

In vitro antiviral activity of T-705 against arenaviruses. The inhibitory activity of T-705 was next examined in vitro against several members of the *Arenaviridae* family of viruses. Against JUNV, PICV, and TCRV, the EC_{50} values were in the range of 5 to 6 μM for T-705 and were 1.7 to 2.2 times less than those obtained with ribavirin (Table 6). Since CPE development in Vero cells exposed to the indicated arenaviruses requires infection of subconfluent cell cultures and extended incubation times, toxic effects associated with drug treatments were amplified. The CC_{50} values ranged from 1,114 to 1,362 μM for T-705 and 156 to 278 μM for ribavirin. The combined greater activity and markedly reduced toxicity of T-705 resulted in SI values that were from 8- to 16-fold higher than those determined for ribavirin (Table 6), compared to a >1.6- to 3.8-fold

TABLE 6. In vitro inhibitory effects of T-705 and ribavirin against arenaviruses[a]

Virus	Strain	T-705[b]			Ribavirin[b]		
		CC_{50} ± SD	EC_{50} ± SD	SI[c]	CC_{50} ± SD	EC_{50} ± SD	SI
JUNV	Candid 1	1,197 ± 337	5 ± 3	239	209 ± 61	11 ± 9	19
PICV	An 4763	1,114 ± 401	6 ± 3	186	156 ± 86	13 ± 9	12
TCRV	TRVL 11573	1,362 ± 197	6 ± 4	227	278 ± 33	10 ± 3	28

[a] Data are the means and standard deviations from three or four separate experiments in Vero cells.
[b] CC_{50} and EC_{50} values are in μM.

TABLE 7. Effects of oral T-705 treatment[a] on hamsters challenged with PICV

Drug	Dosage (mg/kg/day)	Survived/ total	MDD[b] ± SD	Mean virus titer[c,d] ± SD		Mean ALT[c,e] ± SD	Mean liver score[c,f] ± SD
				Liver	Serum		
T-705	60	9/10[i]	22.0	<2.8[h] (0)	<2.8[h] (0)	23 ± 12[h]	0.5 ± 0.0[h]
	30	6/10[i]	12.5 ± 5.7[g]	6.4 ± 1.0[h] (100)	<5.2 ± 1.9 (80)	378 ± 370[h]	0.5 ± 0.0[h]
	15	2/10	11.1 ± 4.2	7.7 ± 0.9[g] (100)	<5.3 ± 1.8 (80)	1,220 ± 926	1.1 ± 0.9
Ribavirin	40	9/10[i]	11.0	<3.2 ± 0.9[h] (20)	<3.0 ± 0.3[h] (40)	30 ± 47[h]	0.0 ± 0.0[h]
0.4% CMC		0/20	8.6 ± 0.8	>9.1 ± 0.3 (100)	5.5 ± 0.1 (100)	2,619 ± 778	2.1 ± 0.4
Sham infected		3/3				25 ± 10	0.0 ± 0.0

[a] Twice daily for 7 days beginning 4 h pre-virus inoculation with 65 PFU of PICV.
[b] MDD of hamsters dying prior to day 28.
[c] Determined on day 7 of infection; five hamsters per group.
[d] Log_{10} $CCID_{50}$/0.1 g of liver or ml of serum. The percentage of animals presenting with detectable virus levels is indicated in parentheses.
[e] Measured in international units per liter.
[f] Score of 0 (normal liver) to 4 (maximal discoloration).
[g] $P < 0.05$ compared to 0.4% CMC-treated controls.
[h] $P < 0.01$ compared to 0.4% CMC-treated controls.
[i] $P < 0.001$ compared to 0.4% CMC-treated controls.

difference for the bunyaviruses (Table 1). The antiviral activity of T-705 was consistent with visual examination of CPE reduction (data not shown) and was verified by virus yield reduction assays (EC_{90} values of 7, 19, and 6 μM for JUNV, PICV, and TCRV, respectively). Thus, the data strongly suggest that T-705 is highly active against the tested arenaviruses in cell-based assay systems and much less toxic than ribavirin.

Treatment of PICV infection in hamsters with T-705. Having seen striking activity against arenavirus challenge in vitro, we sought to determine the efficacy of T-705 in the hamster PICV infection model of severe arenaviral disease. T-705 and ribavirin were given twice per day for a duration of 7 days. As shown in Table 7, the 60-mg/kg/day dose of T-705 was equally as effective (90% protection) as the positive control drug, ribavirin. In fact, the single animal that died survived 22 days, 13 days longer than the MDD of hamsters from the placebo group. A dose-dependent decrease in survival was observed as the dose of T-705 administered decreased. Even the lowest dose of the drug was found to provide significant protection ($P > \chi^2$, 0.002 compared to the placebo group) by log rank survival analysis. Dose responsiveness was also seen with the analysis of viral load and liver disease, with notable improvement even at the lowest dose of T-705. The highest dose of T-705 displayed antiviral activity comparable to that of ribavirin in abrogating the virus burden and liver disease associated with PICV on day 7 of infection.

Therapeutic intervention of PICV infection in hamsters with T-705 and ribavirin. Having demonstrated antiviral activity when treatment was initiated 4 h prior to infectious challenge, we next evaluated the ability of T-705 to protect against PICV infection when given therapeutically. Since delayed ribavirin therapy starting 3 days post-virus inoculation had previously been reported (11), we evaluated the therapeutic effectiveness of equivalent doses of T-705 and ribavirin, with therapy beginning 24 or 72 h after viral challenge. As shown in Table 8, when treatment began 24 h after virus inoculation, the 50-mg/kg/day doses of both T-705 and ribavirin protected all challenged animals from death and comparably reduced the viral burden

TABLE 8. Effects of therapeutic oral T-705 and ribavirin treatment[a] on hamsters challenged with PICV

Drug/start of treatment (h)	Dosage (mg/kg/day)	Survived/ total	MDD[b] ± SD	Mean virus titer[c,d] ± SD		Mean ALT[c,e] ± SD	Mean liver score[c,f] ± SD
				Liver	Serum		
T-705/24	50	10/10[i]	>21	5.7 ± 0.8[h] (100)	<5.1 ± 2.1[g] (80)	61 ± 56[h]	0.2 ± 0.4[h]
	20	2/10[j]	9.5 ± 1.2[h]	>8.4 ± 0.7[j] (100)	>9.0 ± 0.3[j] (100)	1,583 ± 427[h,j]	1.3 ± 0.7[h,j]
Ribavirin/24	50	10/10[i]	>21	4.2 ± 0.9[h] (100)	<3.5 ± 1.0[h] (40)	26 ± 3[h]	0.2 ± 0.4[h]
	20	9/10[i]	18.0	6.0 ± 0.8[h] (100)	<5.1 ± 1.3[h] (80)	24 ± 9[h]	0.0 ± 0.0[h]
T-705/72	50	6/10[i]	14.8 ± 6.1[h]	6.8 ± 1.2[h] (100)	6.6 ± 1.7[g] (100)	411 ± 453[h]	1.2 ± 0.7[h,j]
	20	2/10	8.1 ± 0.6[j]	>8.6 ± 0.7[j] (100)	>8.6 ± 0.2[j] (100)	1,890 ± 579[g,j]	2.0 ± 1.2
Ribavirin/72	50	6/10[i]	18.5 ± 1.9[j]	6.5 ± 0.2[h] (100)	6.0 ± 0.7[h] (100)	15 ± 6[h]	0.0 ± 0.0[h]
	20	7/10[i]	18.3 ± 1.5[h]	6.8 ± 0.4[h] (100)	6.8 ± 0.4[h] (100)	67 ± 33[h]	0.9 ± 0.9[g]
0.4% CMC		0/20	8.1 ± 0.7	>9.5 (100)	>8.8 ± 0.6 (100)	3,042 ± 730	3.0 ± 0.5
Sham infected		3/3				52 ± 3	0.0 ± 0.0

[a] Twice daily for 7 days beginning 24 or 72 h after inoculation with 65 PFU of PICV.
[b] MDD of hamsters dying prior to day 21.
[c] Determined on day 7 of infection; five hamsters per group.
[d] Log_{10} $CCID_{50}$/0.1 g of liver or ml of serum. The percentage of animals presenting with detectable virus levels is indicated in parentheses.
[e] Measured in international units per liter.
[f] Score of 0 (normal liver) to 4 (maximal discoloration).
[g] $P < 0.05$ compared to 0.4% CMC-treated controls.
[h] $P < 0.01$ compared to 0.4% CMC-treated controls.
[i] $P < 0.001$ compared to 0.4% CMC-treated controls.
[j] $P < 0.05$ compared to treatment group receiving equivalent dose of ribavirin.

and liver disease. Ribavirin remained effective (90% survival) at the lower dose of 20 mg/kg/day, whereas the activity of T-705 waned considerably. Nevertheless, the MDD of hamsters treated with the lower dose of T-705 was significantly extended past that of the 0.4% CMC placebo-treated animals. Moreover, log rank survival analysis comparing these two groups indicated a clear beneficial effect ($P > \chi^2$, 0.0003). The 20-mg/kg/day dose of T-705 appeared to slow down the disease process, as suggested by the extended survival times and reduced levels of liver disease (ALT and hepatic icterus) seen on day 7, despite only a 20% survival outcome (Table 8).

When therapy was delayed until 3 days post-infectious challenge, the slight efficacy seen with the 20-mg/kg/day T-705 dose was lost (Table 8). Highly significant 60 to 70% efficacy was seen with the 50-mg/kg/day regimen of T-705 and both doses of ribavirin. These two amounts of ribavirin appeared to be more effective than the high dose of T-705 at slowing the disease process, as reflected by the greatly extended survival times of the animals that died and reduced liver disease present at day 7. The mean ALT values and liver scores for the T-705-treated animals were appreciably higher than for the ribavirin-treated hamsters (Table 8). When viral titers were analyzed, only the high-dose T-705 treatment and both of the ribavirin treatments significantly reduced virus titers comparably, as animals in the 20-mg/kg/day T-705 treatment group had virus loads equivalent to those seen in the placebo-treated hamsters. It is important to note that the LD_{50} for ribavirin (as tested in the hamster PICV infection model) was 217 mg/kg/day, whereas the LD_{50} of T-705 exceeds 1,500 mg/kg/day.

DISCUSSION

The treatment of influenza virus infections has been the primary impetus behind the development of T-705 as an antiviral drug. Recently reported activities against several H5N1 avian influenza A virus strains are encouraging and suggest that T-705 may be an effective alternative for treating outbreaks of pandemic influenza (20). In the initial work describing the anti-influenza activity of T-705, Furuta and others also found some inhibitory activity against poliovirus, rhinovirus, and RSV (7). Here, we have extended the spectrum of T-705, demonstrating robust antiviral activity against a panel of bunyaviruses and arenaviruses in vitro and in vivo with representative rodent models of acute viral infections.

In vitro, we found T-705 to be more potent against the arenaviruses, with EC_{50} values ranging from 5 to 6 μM, while EC_{50} values for the bunyaviruses ranged from 32 to 191 μM. The EC_{50} values seen against the arenaviruses approached those reported against certain influenza A virus strains (EC_{50} values from 0.083 to 3.1 μM), whereas the inhibitory activities against the bunyaviruses were more reflective of those reported for poliovirus (EC_{50} = 31), rhinovirus (EC_{50} = 146), and RSV (EC_{50} = 261) (7). T-705 is metabolized through the activities of cellular enzymes into the active triphosphate form, and the conversion rate for this process likely varies from cell line to cell line (8). Therefore, it is likely that the different cell lines used to assay antiviral activities against the various viruses contributed to the broad range of differences in EC_{50} values. It is also important to note that studies investigating antiviral activities against the arenaviruses were performed in rapidly dividing cells, required for adequate CPE formation by these viruses. These differences, as well as differences in methodologies, make it difficult to directly compare the activities of T-705 against a broad spectrum of viruses.

In relation to the broad-spectrum antiviral ribavirin, we found T-705 to be more effective against the tested arenaviruses and bunyaviruses in our cell-based assays. Ribavirin is reportedly active against more than 16 DNA viruses and over 70 RNA viruses (18). Although much more testing is needed, the spectrum of T-705 appears to be more restricted, since activity against DNA viruses sensitive to ribavirin is lacking (7). The absence of T-705 activity against DNA viruses is likely due to its inability to appreciably alter cellular DNA (or RNA) synthesis and its markedly reduced capacity to inhibit IMPDH, both of which are proposed mechanisms of action that likely contribute to the toxicity of ribavirin (23).

Although there were some cases where subtle differences in efficacy between T-705 and ribavirin were evident, essentially they were equally effective in treating PTV infection in mice and hamsters. Interestingly, our data showed that lower doses of ribavirin were more effective than T-705 for the treatment of PICV infection. We previously showed that 20 mg/kg/day oral ribavirin is the lower limit for optimal protection against PICV infection when treatment is initiated 24 h post-infectious challenge (11). Here, we have verified that finding and reported that 50 mg/kg/day was the lowest tested dose of T-705 that provided complete protection in the PICV infection model. Considering the toxicity of ribavirin in hamsters (LD_{50} = 217 mg/kg/day) and the lack of toxicity with T-705 (LD_{50} > 1,500 mg/kg/day), the latter may be a viable alternative for arenaviral disease. The results of our studies with T-705 are very encouraging in that ribavirin is the only antiviral drug indicated for use in cases of Lassa fever. Ribavirin therapy can lead to reversible hemolytic anemia, and the potential for adverse effects, primarily in pregnant or lactating women, continues to be a concern (23).

Despite proven activity against RVFV in rodent and nonhuman primate models (16), ribavirin has yet to be thoroughly evaluated for the treatment of RVFV infection in humans. Notably, a small-scale, randomized, placebo-controlled clinical trial evaluating ribavirin for the treatment of severe RVFV infections was conducted during the Saudi Arabia outbreak of 2000 (3). Although not conclusive, an increased incidence of the encephalitic form of the disease was observed in cases treated with ribavirin (P. Rollin, presented at the Treatment of Viral Hemorrhagic Fever Workshop, Bethesda, MD, 24 to 27 February 2007). In this regard, our studies demonstrating robust activity of T-705 against PTV infections modeling severe RVFV infections are very encouraging. In contrast to the results with the PICV infection model, comparable lower limits of protection were seen with both drugs when tested against PTV infections in mice and hamsters. Interestingly, the toxicity of T-705 in mice was greater than that observed in hamsters by the tested route and schedules. The opposite result was observed with ribavirin, and it is conceivable that its reduced toxicity in mice may be due in part to the shorter treatment duration (5 days) than that of hamsters (7 days). Nevertheless, the LD_{50} of ribavirin in 12- to 14-g C57BL/6 mice (730 mg/kg/day) was unexpectedly high, since its administration by the same route and at the same frequency and duration in 17-g

female BALB/c mice had a reported LD_{50} of 220 mg/kg/day (19). We suspect that the difference in ribavirin toxicity is a result of the species and possibly age differences in the mice used.

Pharmacokinetic analysis of T-705 in mice indicates that only 10% of the drug remains 6 h following a single p.o. administration (Toyama Chemical Co., Ltd., unpublished data). As a consequence, initial influenza virus studies with T-705 employed four-times-daily treatment schedules (7, 24). Since the added stress on weanling mice due to such a rigorous treatment schedule would have been less than ideal, we decided that it would be best to evaluate T-705 given twice daily. Doing so reduced handling stress and facilitated comparative studies with ribavirin, commonly given twice daily. Moreover, several of us had observed efficacy against lethal avian influenza A virus infection with twice-per-day, once-per-day, and even single-dose treatment schedules (20). The once-per-day treatment and single-dose therapeutic interventions were not explored in the PTV and PICV infection models, so it is unclear whether adequate protection would have resulted. Studies investigating this matter with T-705 and ribavirin are under way.

T-705–ribofuranosyl-5′-triphosphate (T-705RTP) acts primarily through highly specific inhibition of the influenza RNA polymerase with little disruption of IMPDH activity by the monophosphate form, T-705RMP (8). Although the RNA polymerases of other RNA viruses, including arenaviruses and bunyaviruses, are the likely targets of T-705RTP, studies are needed to verify this primary mechanism of action. The ability of T-705 to protect infected mice with infrequent dosing, in spite of the pharmacokinetic data, which indicate >90% clearance by 6 h post-oral administration, suggests that the drug persists intracellularly for extended periods and/or that it may act as an immune response modifier (20). The applicability of T-705 for the treatment of viral infections in the brain remains to be seen. This is of particular interest for encephalitic viruses, such as LACV. Studies with T-705 are under way to address many of these questions related to delineating additional modes of action, intracellular persistence, and tissue distribution following treatment.

Currently, T-705 is in the early stages of phase I clinical trials in Japan and the United States for the treatment of influenza virus infections. Clinical toxicologic and pharmacologic assessments will be forthcoming. Our findings are most encouraging and indicate strong potential for T-705 as a therapeutic for the treatment of arenaviral and bunyaviral infections in humans and, in the case of RVFV, in livestock. Even so, it is difficult to define in cell-based and rodent model systems how much better a drug will have to be to overcome the shortcomings of ribavirin, the only currently indicated treatment for the aforementioned viral infections. Certainly, reduced toxicity and efficacy comparable to that of ribavirin in rodent model systems make T-705 a promising drug candidate. Considering the bioterror threat that viruses related to those included in our studies pose, further investigations examining the antiviral activity of T-705 against bona-fide NIAID category A arenaviruses and bunyaviruses, in appropriate high-containment settings, is imperative.

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